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Development, production, purification, and activity of recombinant lamprey gonadotropin in S2 Drosophila and Chinese hamster ovary cell lines

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DEVELOPMENT, PRODUCTION, PURIFICATION, AND ACTIVITY OF RECOMBINANT LAMPREY GONADOTROPIN IN S2 DROSOPHILA AND CHINESE HAMSTER OVARY CELL LINES

BY

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DEDICATION

To My Family and Marni

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ABSTRACT

DEVELOPMENT, PRODUCTION, PURIFICATION, AND ACTIVITY OF RECOMBINANT LAMPREY GONADOTROPIN IN S2 DROSOPHILA AND CHINESE HAMSTER OVARY CELL LINES

By

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University of New Hampshire, September, 2008

The objective of this research was to develop methods for the production and purification of recombinant lamprey GTH beta. The overall goal was to produce an active purified recombinant lamprey GTH that could be used for immunological, physiological, and histological studies to further understand the role of the gonadotropin in the sea lamprey, *Petromyzon marinus*. Constructs with different combinations of tethered lamprey gonadotropin beta to human CG alpha and channel catfish gonadotropin alpha were transfected and expressed in stable cell lines of Drosophila (S2) cells and Chinese hamster ovary (CHO) cells. Cell lines were grown at a large scale and protein was collected for purification. Expression of the gonadotropin was tracked through all phases of the scale-up using RT-PCR in conjunction with Western Blot. Initial purification used a combination of nickel sepharose purification for 6XHis tagged product and size exclusion chromatography for untagged products. Activity of concentrated purified fractions was determined through the use of *in-vitro* culture of lamprey gonadal tissue, as well as *in-vitro* receptor activity cAMP assays from transiently transfected COS-7 cells. Attempts to combine lamprey GTH beta with other alpha

subunits through coexpression and tethered constructs did not increase yield or produce an active product. It is likely that the lamprey GTH alpha will be required in order to conform to the beta subunit for functional protein activity. The results from this study should be used to develop procedures for production of recombinant lamprey gonadotropins and will be especially important once the alpha subunit has been identified.

CHAPTER I

BACKGROUND AND SIGNIFICANCE

HYPOTHALAMAL-PITUITARY GONADAL AXIS

A key neuroendocrine function of the hypothalamus in the control of reproduction is the release of the decapeptide gonadotropin-releasing hormone (GnRH) that acts on the pituitary gland. GnRH is the major brain hormone that controls reproduction in all vertebrates (Gorbman and Sower 2003). In gnathostomes, GnRH is released from the hypothalamus and activates receptors in the pituitary to stimulate the release of two gonadotropins (GTH), luteinizing hormone (LH) and follicle stimulating hormone (FSH). Both hormones stimulate steroidogenesis and gametogenesis at the ovaries or the testis, leading to reproductive maturation.

GnRH is a highly conserved decapeptide. All GnRH forms have conserved amino and carboxyl terminals, $pGlu^1$ and $Pro^9Gly^{10}NH_2$, respectfully, as well as a conserved Ser in the fourth position. The sixth position, containing Gly^6 is considered critical in maintaining the β-turn, which forms a horseshoe structure for receptor binding of GnRH in mammals (Sealfon et al., 1997). There are twenty-six different isoforms, fifteen of which are found in vertebrates (Kavanaugh et al., 2008; Zhang et al., 2008). The GnRHs identified in mollusks, Octopus and Aplysia, are the only two non-chordates found to have GnRH, and the only ones identified that have twelve amino acids (Iwakoshi et al., 2002; Zhang et al., 2008). In lamprey, three different isoforms of GnRH (lamprey GnRH-I, -II and -III) have been identified and characterized (Kavanaugh et al., 2008; Sherwood et al., 1986; Sower et al., 1993). Lamprey-I and -III are both considered to be hypothalamic GnRHs acting on the pituitary-gonadal axis (Sower 2003). Lamprey GnRH-I or III has been shown to have varying effects on steroidogenesis and spermiation/ovulation in adult lampreys (Deragon and Sower 1994; Gazourian et al., 1997; Gorbman and Sower 2003; Sower 1990; Sower 2003; Sower et al., 1993). It is also interesting to note that the primary amino acid sequences of lamprey GnRH-I and III varies in the sixth position compared to all other vertebrate GnRHs, replacing glycine with glutamate and aspartate, respectively (Figure 1).

Vertebrate	1	2	3	4	5	6	7	8	9	10
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	GlyNH2
Guinea Pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	GlyNH2
Chicken - I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	GlyNH2
Rana	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	GlyNH2
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	GlyNH2
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	GlyNH2
Whitefish	pGlu	His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	GlyNH2
Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	GlyNH2
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	GlyNH2
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	GlyNH2
Chicken -II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	GlyNH2
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	GlyNH2
Lamprey - II	pGlu	His	Trp	Ser	His	Gly	Trp	Phe	Pro	GlyNH2
Lamprey - III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	GlyNH2
Lamprey - I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	GlyNH2

Figure 1: The GnRH family of peptides in vertebrates. Identified are the key residues that vary between the peptides. To date, there are 15 identified GnRH's in vertebrates. (Kavanaugh et al., 2008)

Recently, a third form of GnRH (lamprey GnRH-II) has been identified in lamprey. Through initial immunocytochemistry, *in situ* hybridization and biological studies, it has been suggested to also act as a hypothalamic GnRH (Kavanaugh et al., 2008). Similar to the other vertebrate GnRHs but not lamprey GnRH-I or III, lamprey GnRH-II has a glycine in the sixth position (Kavanaugh et al., 2008).

In gnathostomes, GnRH stimulates reproduction via the hypothalamic-pituitary-gonadal axis stimulating the release of the gonadotropins, LH and FSH, through the interaction of GnRH with its receptor in the pituitary. In lampreys, GnRHs also control reproduction via the pituitary-gonadal axis; however lampreys only have one gonadotropin instead of the two gonadotropins, LH and FSH, found in gnathostomes (Sower et al., 2006).

GONADOTROPINS

Gonadotropin hormones have been isolated in numerous species of vertebrates, including *Danio rerio* (zebrafish)(Kwok et al., 2005), *Hippoglossus hippoglossus* (Atlantic Halibut) (Weltzien et al., 2003) and a species of mouse (*Praomys coucha*) (Takano et al., 2004) (Figure 2). There are three major types of pituitary glycoprotein hormones present in gnathostomes: LH, FSH, and thyroid stimulating hormone (TSH). LH and FSH are responsible for reproductive development and growth across vertebrates. TSH acts via thyroid hormones to regulate metabolism. There is one other glycoprotein, human chorionic gonadotropin (hCG), present only in placental mammals and only during pregnancy. Two gonadotropins, (LH and FSH) have been identified in representative species of all taxonomic groups of gnathostomes. In lampreys, only one gonadotropin beta (GTH-β) subunit has been identified (Sower et al., 2006). Figure 2 shows the identification of the various glycoprotein hormones in vertebrates.

Glycoprotein Hormones in Vertebrates			
	LH	FSH	TSH
Mammalia	Human,Cattle,Pig Rat,Horse,Sheep, Opossum	Human,Cattle,Pig Rat,Horse,Sheep, Opossum	Human, Cow, Pig, Dog Rat, Mouse, Opossum
Aves	Ostrich, Quail	Ostrich, Quail	Quail, Chicken, Duck, Ibis
Reptilia	Turtle, Alligator, Snake	Turtle, Alligator, Snake	Turtle, Alligator, Snake
Amphibia	Bullfrog	Bullfrog	Xenopus, Bullfrog
Dipnoi	*Lungfish*	*Lungfish*	*Lungfish*
Actinopterygii	Sturgeon, Eel, Carp, Goldfish, Salmon, Tuna, Bonito, Yellowtail, Croaker, Killifish, Sea bream	Sturgeon, Eel, Carp, Goldfish, Salmon, Tuna, Bonito, Yellowtail, Croaker, Killifish, Sea bream	Sturgeon, Zebrafish, Rainbow trout, Eel, Goldfish, Salmon
Chondrichthyes	Catshark	Catshark	+
	GTH		
Agnatha	Hagfish (unpubl.), Sea lamprey (2006)		

Figure 2: A schematic diagram of most of the identified glycoprotein hormones in vertebrates: LH, Luteinizing hormone; FSH, Follicle stimulating hormone; TSH, Thyroid stimulating hormone; and GTH, Gonadotropin hormone (Kawauchi, 2005).

Functions and/or activities of the GTH hormones are based on several specific physical properties and modifications. The protein itself exists as a heterodimer consisting of an alpha and a beta subunit. The alpha subunit for LH, FSH, and TSH is identical in any one species of vertebrate. This highly conserved subunit has a total of 10 cysteine residues that form five disulfide bonds which identify the tertiary structure of the subunit (Figure 3). Crystal structures of the human alpha subunit suggest that the disulfide bonds exist at these 10 specific key residues: 7-31, 59-87, 10-60, 28-82, 32-84 (Sato et al., 1997). The last 3 disulfide bonds define the cysteine knot formation within the protein, which is crucial in protecting the protein from degradation through proteolytic enzyme cleavage. It allows the protein to pack tightly by excluding water and making an extremely hydrophobic region, improving amino acid interactions and internal hydrogen bonding. The cystein knot is important in providing integrity of the protein, and in signaling for the protein's secretion from the cell (Sato et al., 1997).

The beta subunit of GTH is not identical across any one species, having variability in its overall structure from early vertebrates (lamprey) to humans, and it determines the specificity of the action of the glycoprotein. All beta subunits consist of 12 cysteine residues that create six disulfide bonds. These bonds are important for hormone assembly and secretion from the cell (Suganuma et al., 1989), but are not required for hormone activity (Ben-Menahem et al., 1997).

Downstream regulation of gonadotropin (LH and FSH) activity at the gonads is shown to be mediated by two major signaling pathways; cAMP and Inositol triphosphate (IP3) as second messengers (Gudermann et al., 1992). These GTH signaling pathways are mediated through a subfamily of G-protein coupled receptors (GPCRs), or more specifically glycoprotein hormone receptors (GpH-Rs) (Combarnous 1992). The extracellular N-terminal domain of the receptor is responsible for high affinity binding of the hormone. The C-terminal portion contains the transmembrane region with the seven typical alpha helices as well as the intracellular domain (Grossmann et al., 1997a; Moyle et al., 2005). Although the IP3 signaling pathway has been shown to play a role in receptor activity, the major signaling pathway for gonadotropin receptors is believed to be the cAMP pathway (Gudermann et al., 1992).

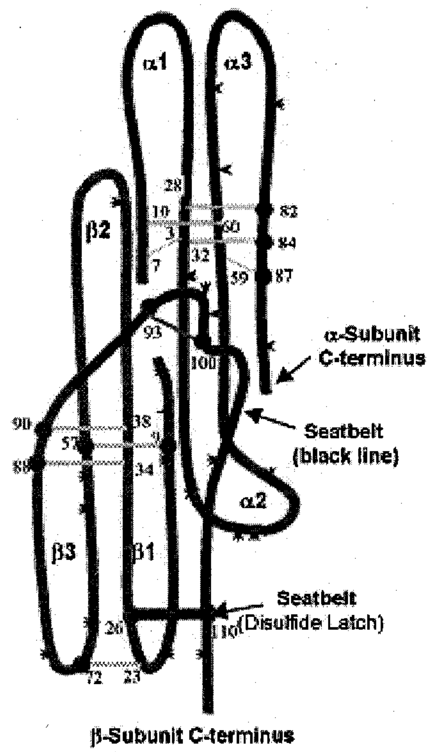


Figure 3: A schematic showing the overall structure and conformation of the alpha and beta subunit of the gonadotropin. Identified also are the key cysteine residues responsible for forming the characteristic cysteine knot, as well as the seatbelt region of the beta subunit responsible for heterodimer formation (Moyle et al., 1998).

LAMPREY GONADOTROPIN

Recently in lamprey, the GTH-β subunit was isolated and its amino acid structure determined through a long-term collaboration of Professors' Sower and Kawauchi laboratories (Sower et al., 2006). It was hypothesized from the studies that lamprey have only one pituitary glycoprotein, a gonadotropin-like hormone. The GTH-β like DNA was cloned from a plasmid library. A mature protein of 134 amino acids with a 15 amino acid signal peptide was identified. The specific 16 amino acid GTH-β peptide was used for antibody production. Using immunohistochemistry (IHC), the lamprey

GTH-β like protein was shown to be expressed in the PPD (proximal pars distalis) of the lamprey pituitary. Administration of lamprey GnRH I or III increased expression of GTH-β in the pituitary as determined by real-time PCR (Sower et al., 2006). The alpha subunit has not yet been determined, but an alpha-like subunit has been identified from the lamprey genome, and full sequencing should be done within the next year. Positive identification of the lamprey gonadotropin has now opened many possibilities for future reproductive studies.

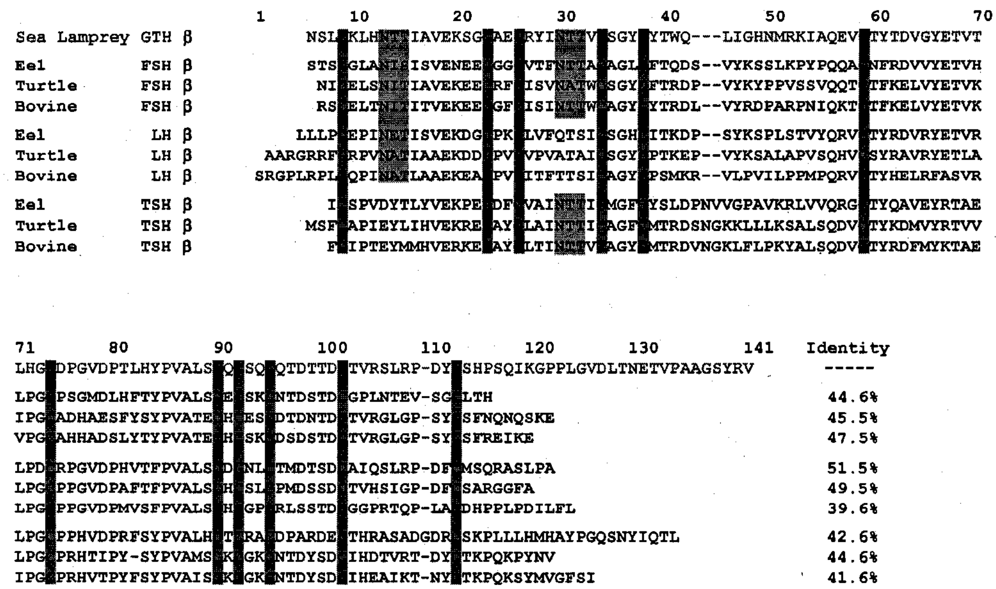


Figure 4: A comparison of the lamprey gonadotropin beta subunit to three other known species of LH, FSH and TSH. Identified in red are the twelve cysteine residues representative of all the gonadotropins, and labeled in green are possible sites for post translational modification (glycosylation)(Sower et al., 2006).

When lamprey GTH-β is compared to the beta subunit in other vertebrates, it shows a high percent identity to other glycoproteins (TSH, FSH, LH), when compared to eel, turtle, and bovine (Figure 4). When comparing the protein sequence to other fish species, lamprey GTH-β shows high percent identities to newt (49%) and carp (51%) LH-β subunits. Conversely, it shows a much lower percent identity against other FSH and TSH species, suggesting that the subunit is a LH-like hormone. Analysis of the vertebrate lineage of GTH-β subunits identifies the lamprey GTH-β as an out-group to the rest of the vertebrate line (Sower et al., 2006). The identification of only one GTH has led many to believe that after the agnathans, gene duplications or proposed whole genome duplications gave rise to the multiple glycoproteins.

A key factor in gonadotropin hormone activity is the proper glycosylation of each subunit. On each of the alpha and beta subunits there exist three possible sites for N-linked glycosidic chains. Although the actual role of the glycosidic chain in the association of the hormone to the receptor is still unknown, glycosylation is considered necessary for several functions. As an example, studies showed that glycosylated compared to non-glycosylated FSH improved the activity of the hormone by increasing its half-life in vivo (Bishop et al., 1995). The N-linked oligosaccharides and O-linked oligosaccharides comprise 18-45% of the total protein weight (Combarnous 1992). By weight, the equine chorionic gonadotropin (eCG) contains the most glycosylation of the glycoprotein hormone family, consisting of 45% carbohydrates (Gospodarowicz 1972; Harbon-Chabbat et al., 1961). These moieties do not apparently interfere with receptor binding, since the sites of glycosylation are located far from the receptor affinity sites (Combarnous 1992). However a lack of these glycosidic residues leads to inactivity of the protein and thus inactivity in the cell, therefore suggesting the importance of the glycogen moieties in gonadotropin production and isolation.

LAMPREY GLYCOPROTEIN HORMONE RECEPTOR

Recently, two glycoprotein hormone receptors (GPH-R) were cloned from lamprey (Freamat et al., 2006; Freamat and Sower 2008). Both receptors were cloned from cDNA isolated from the adult sea lamprey. The full length transcripts encode two functional lamprey receptors; Lamprey GpH-R I (lGpHR-I) of 719 amino acids, and Lamprey GpH-R II (lGpHR-II) of 781 amino acids. Lamprey GpH-R I was shown to be more highly expressed in the testes, whereas lamprey GpH-R II was more highly expressed in the thyroid. Both receptors were expressed in other tissues, but at lower levels. The sea lamprey glycoprotein hormone receptors were also shown to activate the cAMP signaling pathway, and thus were determined to be functional receptors (Freamat et al., 2006; Freamat and Sower 2008).

Lamprey GpHRs have many similarities to glycoprotein receptors from other vertebrates, but have several differences when compared to each other. Lamprey GpHR-I has the most similarity to luteinizing hormone receptors (LHR), and lamprey GpHR-II is most similar to thyroid stimulating hormone receptors (TSHR). One notable difference is the presence of an abnormally long linker domain (SSD) in the lGpH-R II, varying greatly from the lGpH-R I which has the shortest SSD domain of all vertebrates (Freamat et al., 2006). This extended SSD region between the leucine rich domain (LRD) and transmembrane domain (TMD) is 50 amino acids in length, and is characteristic of other thyrotropin receptors in vertebrates (Farid and Szkudlinski 2004). The function of the SSD domain is not established, however Moyle et. al. (2005) has proposed that the SSD has a significant role in ligand binding and signaling.

RECOMBINANT PROTEIN PRODUCTION

Due to the low overall production of glycoproteins in fish species, purification of gonadotropins in large quantities has become a major issue. Production of protein using recombinant methods allows for greater yields of similar protein products with an increased yield over natively produced proteins. The definition of a recombinant protein is any protein produced using cellular machinery derived from an expression system (*in-vivo* or *in-vitro*). Generally the protein that is expressed is not native to the cell type or expression system that is utilized. The selection of the proper expression system ultimately decides the amount of protein that can be produced. An expression system combines a vector containing the recombinant DNA of interest with a specific cell line or cell type. Cell that are the most stable are usually immortalized cells, and can be derived from many different types of organisms (Wurm 2004). The cell line that is chosen reflects the type of protein that is produced, and therefore should be optimized to include all structural similarities that are necessary for the functional protein (Wurm 2004).

YEAST AND BACTERIAL EXPRESSION

Large scale protein production using yeast and bacterial cell lines is useful when trying to purify large quantities of non-complex proteins, such as those with minimal folding specific to activity, and low levels of post translational modifications (e.g. glycosylation). Replication of both cell types is extremely rapid, and can readily grow in large scale suspension cultures. Problems arise when producing complex proteins that require specific folding and glycosylation complexes to retain activity (Gasser et al., 2007). Yeast (*Saccharomyces cerevisiae*) cell lines that produce complex proteins, have been hindered by the lack of protein secretion ability (Valkonen et al., 2003). Modifications to the promoters and fusion to certain signal proteins have helped to improve the secretory pathway in yeast (Ruohonen et al., 1995), but not without significant genetic manipulation of each cell line. Thus, these are not useful cell lines for proteins such as the highly glycosylated glycoprotein hormones and others that require a more extensive post translational modification.

BACULOVIRUS EXPRESSION

A system that can be used to produce large quantities of highly glycosylated proteins involves the use of a lytic viral expression system. Systems developed using baculovirus infections of *Spotoperda frugiperda* (SF-9) and Chinese Hamster Ovary (CHO) cells have been successful in producing active recombinant gonadotropins that retain extensive glycosylation (Grossmann et al., 1997b). Although the patterns of mammalian cell glycosylation and insect cell glycosylation differ in final processing steps (Hsieh and Robbins 1984), the resulting gonadotropin products still retain substantial biological activity (Grossmann et al., 1997b).

Although the baculovirus system has the potential to yield high quantities of the necessary product, expenses are higher in maintaining such a cell line. Since the baculovirus expression of protein uses a lytic system, development of a stable cell line is not possible. The entire expression process must be repeated after infection and cell lysis. It requires the growth of separate viral stocks and manipulation of the viral DNA in order to incorporate the gene of interest (Legardinier et al., 2005). The baculovirus expression system remains a good option for large scale production of biologically active hormones, although it is much more costly.

DROSOPHILA EXPRESSION SYSTEM

The drosophila expression system, or DES, has been shown to yield recombinant gonadotropins that are highly specific due to the folding patterns and post-translational modifications obtained through insect cells (Shoham and Insler 1996). The Drosophila system is the most specific and inexpensive way to produce recombinant proteins, due to its minimal growth conditions and extensive protein glycosylation patterns (Combarnous 1992). Insect cell protein glycosylation is one of the most intricate, second only to mammalian cell lines. This has led to high success in the production of glycoproteins and gonadotropins which all have a need for proper glycosylation (Ivey-Hoyle 1991; Yokomizo et al., 2007; Zmora et al., 2003). The cells can be grown at room temperature, optimally between 25-28^{o}C and have no CO_2 requirements unlike many cell lines. Cells may also be recovered after media collection and expanded further with minimal cell death, as opposed to the more involved lytic bacteriophage system of the baculovirus production scheme. The ability to develop a stable cell line that can be stored for long term use makes this system the ideal candidate for recombinant gonadotropin production. Therefore, it was decided to test the DES expression system in the production of recombinant lamprey GTH. Refer to Figure 5 for a brief comparison of the requirements and protein complexity of each different cell type.

Bacterial Cells	Yeast Cells
-Inexpensive, High Yield -Does not produce proper folding or glycosylation -Final protein is non functional	-Incapable of producing complex proteins -Only modified yeast cells can produce proper glycosylation
Insect Cells	**Mammalian Cells**
-Glycosylation similar to that of mammalian cells -May not be suitable for production of functional mammalian proteins -Inexpensive, Easiest to culture, little requirements -High yield, easy purification	-Protein glycosylation is even more specific -Proper folding and attachment of extracellular components -Most expensive -Low yield

Figure 5: Brief comparisons of different expression systems for recombinant protein production (Legardinier et al., 2005; Shoham and Insler 1996; Suzuki et al., 2006; Valkonen et al., 2003; Wurm 2004)

CHIMERIC HORMONES

The expression of recombinant hormones in any cell line can be manipulated to produce one or several proteins at a time, depending on what is transfected into the cells. In some cases, production of multiple subunits is necessary to increase yield and secretion of the protein from the cell. In the case of gonadotropins, production of both the alpha and beta subunit is necessary for hormone activity. The common alpha and beta subunit have specific disulfide bonds that are required for dimerization of the two subunits (Pierce and Parsons 1981), since disruption of the disulfide bonds or chemical reduction of either subunit inhibits assembly (Suganuma et al., 1989). In order to maintain close complex relationships with subunits, they can be modified to be expressed as one, and still allow for proper folding of each subunit (Sugahara et al., 1995).

An example of enhanced activity through chimeric protein production is the production of recombinant hCG that has been modified to alter the interaction between the alpha and beta chains. When the alpha subunit "seatbelt region" is modified, it can have the ability to interact with the beta subunit to initiate a disulfide bond in a modified region of the protein, allowing for increased LH activity (Xing et al., 2001). The placement of the disulfide bond will determine whether it increases or decreases activity of the protein heterodimer (Xing et al., 2001).

A second example of direct chimeric hormone production was the production of biologically active equine LH, which consisted of both alpha and beta subunits linked directly using overlapping PCR (Jablonka-Shariff et al., 2007). This was developed due to the problem of producing both subunits separately in CHO cells. When the two hormones were combined as one protein before translation, the problem of subunit association was eliminated, and protein expression was increased.

TETHERED HORMONES

Protein interactions can be increased by forcing them to be closely related when produced from the cell. In the case of tethered proteins, amino acids can be added between two proteins that will not interfere with protein/protein interactions, but can increase protein activity with the receptor (Garcia-Campayo et al., 1997). Modifications that can help maintain proper folding of each subunit include the use of a tethered chain of amino acids as a linker. In the case of the gonadotropins, the beta subunit of hCG has a characteristic unique from other beta subunits. A region on the carboxy-terminal end has four serine-linked oligosaccharides, called the CTP (carboxy-terminal-peptide) region of the hormone. In hCG, these oligosaccharides aid in increasing the half-life of the hormone in-vivo and are crucial to in-vivo potency, but is not critical for receptor binding (Matzuk et al., 1990). This region acts as a suitable linker since it is serine/proline rich and thus lacks a predominant secondary structure (Kanda et al., 1999). Due to the small size and negligible charge characteristics of amino acids, a glycine/serine repeat would also be suitable as a protein linker (Kasuto and Levavi-Sivan 2005).

Since only one gonadotropin subunit has been identified in lampreys, several tethered constructs were made using this linker to attach either channel catfish GTH-α or human CG-α to the lamprey GTH-β subunit. These GTH alpha subunits were chosen based on comparisons of the lamprey GTH-β subunit structure with other beta subunits. In addition, Dr. William Moyle (Robert Wood Johnson Rutgers Medical School, Piscataway, NJ) did further in-depth analysis to examine differences in the way the lamprey beta subunit may be able to interact with an alpha subunit. Through amino acid comparisons of gonadotropins from various vertebrates, it is believed that the beta subunits interact with the alpha with one of two overall schemes, "wraparound" and "threading" (Moyle et al., 2005). The diagram below displays the differences between the teleost and other vertebrate gonadotropin dimer formation (Figure 6). Structure of the lamprey gonadotropin beta subunit is more similar to human and other vertebrates than to teleost beta subunit folding patterns. Therefore, both possible interactions were tested, through the production and purification of recombinant tethered gonadotropins prepared with lamprey GTH-β and human CG-α. The alpha subunit of channel catfish GTH, a teleost, was also tested to determine if lamprey GTH-β could be tethered with an alpha subunit from osteichthyes.

Models of Heterodimer Formation

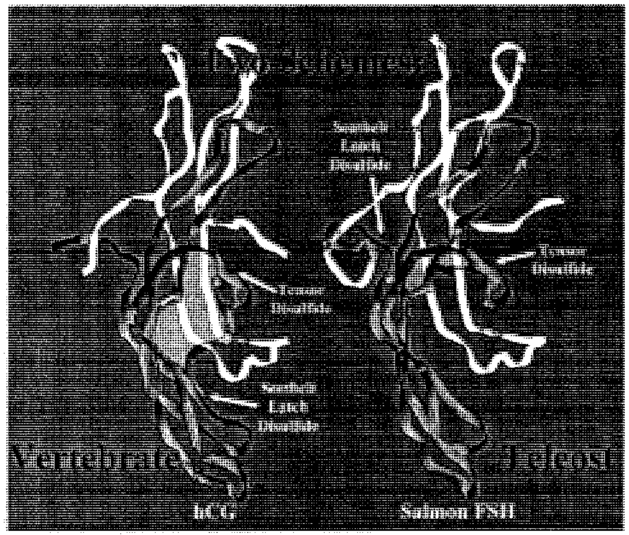


Figure 6: Two models of glycoprotein hormone heterodimer formation. This shows the difference in conformation between the vertebrate and teleost seatbelt loop region, pointing out major differences with hormone heterodimer interaction. The vertebrate scheme of alpha subunit interaction involves the "wraparound" of the NH2 terminus of the beta which has a larger opening and a weaker interaction. The smaller hole of the teleost FSH-β gives the heterodimer more protein stability, and allows for the threading of the NH2 terminal of the beta subunit forming the "seatbelt latch noted above (Xing et al., 2004). Lamprey GTH-β is believed to follow the vertebrate form of subunit interaction based on structural analysis by Professor Moyle, Robert Wood Johnson University, New Brunswick, NJ.

LAMPREY LIFE CYCLE

Lampreys are members of the class Agnatha, which are jawless fish that evolved along with hagfish over 550 million years ago (Janvier 1981). There are approximately 40 species of lampreys and depending on the species, they can be parasitic or non-parasitic. The sea lamprey, *Petromyzon marinus*, is parasitic and the largest species of all the Agnathans. The sea lamprey, being anadromous, lives part of its life in freshwater and part in seawater. Following fertilization of the eggs in freshwater, the synchronized seasonal cycle begins as the embryos develop into larval lamprey called ammocoetes. Ammocoetes live as sediment feeders and burrow into the sand of coastal rivers and streams for five to seven years of their life until reaching the metamorphic stage (Hardisty 1971). At this stage the ammocoete lacks functional eyes, gill slits, and the distinctive mouth of teeth (Hardisty 1971). During the process of metamorphosis, larval lampreys develop into a free-swimming, parasitic lamprey that will migrate from the streams to the ocean. The eyes and mouth have now developed, even though they remain sexually immature .

Not only does the lamprey undergo major morphological metamorphosis, but it also exhibits major physiological changes. Following the migration from freshwater to the ocean, the lamprey begins its stage of life that is parasitic. During this 15-month ocean phase, the sexually immature lamprey feeds on fish by attaching to the fish with its oral hood and teeth, sucking the blood and other tissue from the fish (Hardisty 1971). At the end of this period the lamprey migrate back to freshwater streams to complete sexual maturation (Hardisty 1971). This results in the development of the ovaries in females and the testes in males. Once ovulation occurs and the sperm has matured, the lamprey will spawn and then die (Hardisty 1971).

Each stage of the life cycle represents a specific change in the gonads for each fish. During the initial larval stage, the ammocoetes, male and female, only have primary germ cells and primary oocytes (Sower 2003). Within the next year, metamorphosis causes the initial mitotic division in females. In males, this also occurs along with the development of nests containing primary spermatogonia. While in the parasitic phase, the immature lamprey progress into gametogenesis. During this time, the females undergo prophase of meiosis I and yolk deposition. Vitellogenesis in females occurs while males testes begin to grow, leading to the mitotic divisions of the primary spermatogonia and producing some secondary spermatocytes (Nozaki et al., 1999; Sower 2003). At the end of the parasitic stage and during the return to freshwater, the male and female lampreys undergo final maturation of the gonads and prepare for spawning. Females experience an extremely rapid growth of gonadal tissue and produce a large number of eggs ranging from 124,000 to 260,000 in landlocked *Petromyzon marinus* (Gorbman and Sower, 1999). Secondary sexual characteristics also develop in both sexes during this time including an enlargement of the fins and the fusion of both dorsal fins into one (Gorbman and Sower 1999). Overall, sexual maturation and the development of the gonads in lamprey are facilitated by the two major isoforms of GnRH, which control the pituitary-gonadal axis (Sower 2003)(Figure 7).

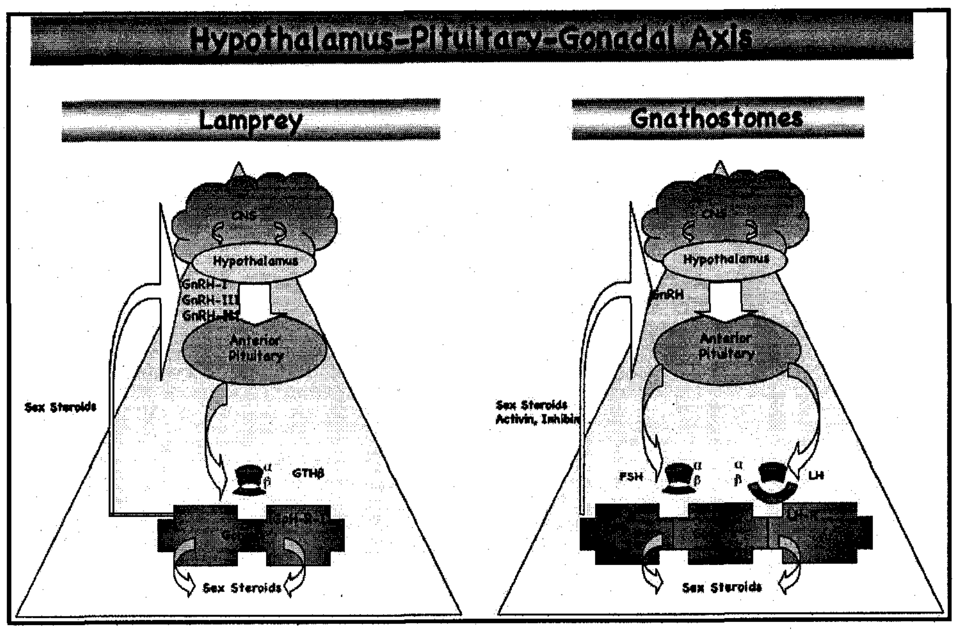


Figure 7: A diagram comparing the lamprey versus the gnathostome hypothalamic-pituitary gonadal axis. In lamprey, the release of GnRH-I, and -III (possibly –II) from the hypothalamus acts directly on the pituitary, causing the release of GTH from the anterior pituitary, which acts on one receptor at the gonads to stimulate reproduction (Sower et al., 2008). In gnathostomes, GnRH acts on the anterior pituitary, which releases the gonadotropins (FSH and LH) that act on specific receptors at the gonads to stimulate reproduction.

PROJECT SUMMARY

The objective of this research was to develop methods for the production and purification of recombinant lamprey GTH-β. The overall goal was to produce an active purified recombinant lamprey GTH that could be used for immunological, physiological, and histological studies to further understand the role of the gonadotropin in the sea lamprey, *Petromyzon marinus.* Constructs prepared with different combinations of tethered lamprey gonadotropin beta to human CG alpha and channel catfish gonadotropin alpha were transfected and expressed in stable cell lines of insect Schneider's (S2) cells and mammalian Chinese Hamster Ovary (CHO) cells. Large scale cultures of cell lines were grown and protein from the growth medium was collected for purification. Expression of the recombinant gonadotropin was tracked through all phases of the scale-up using RT-PCR as well as Western Blotting of protein product. Initial purification used a combination of nickel sepharose purification of a His tag product and size exclusion chromatography for untagged products. Activity of concentrated purified fractions was determined through the use of *in-vitro* culture of lamprey gonadal tissue, as well as *in-vitro* receptor activity cAMP assays from transiently transfected COS-7 cells.

CHAPTER II

PRODUCTION AND PURIFICATION OF TETHERED LAMPREY RECOMBINANT GONADOTROPINS

INTRODUCTION

Gonadotropins (GTH) are pituitary glycoprotein hormones consisting of FSH (follicle stimulating hormone), and LH (luteinizing hormone). TSH (thyroid stimulating hormone) and CG (chorionic gonadotropin) are also glycoprotein hormones, but are found in the thyroid of vertebrates and placenta in mammals respectively. Gonadotropins have been isolated and purified in many species of vertebrates, including salmonids (Swanson et al., 1991), rat (Chin et al., 1983), mouse (Kumar and Matzuk 1995), and chicken (Shen and Yu 2002). Only recently has the GTH-β subunit from sea lamprey been isolated and its tertiary structure determined (Sower et al., 2006). The identified sea lamprey preGTH-β cDNA encoded 150 amino acids, in which the mature lamprey GTH-β consists of 134 amino acids (Sower et al., 2006). With twelve cysteine residues representative of the gonadotropin hormone cystein knot and two N-glycosylation sites, the lamprey GTH-β showed similar homology to all three glycoprotein hormones (TSH, FSH, LH). A comparison against other gnathostome species showed the highest percent identity of lGTH-β to Eel LH-β, although the third glycosylation site is more similar to CG. To fully examine the function of lamprey GTH, it is necessary to have sufficient quantities of purified protein. Therefore the objective of this study was to develop and refine methods for the production and purification of recombinant lamprey GTH.

The low abundance of gonadotropins in fish pituitaries has made it difficult to purify large quantities of native gonadotropin from tissue that is necessary to use for antibody production and functional studies (Zmora et al., 2003). The availability of GTH is critical for further immunological, physiological, and histological studies; therefore the majority of efforts in GTH synthesis have used the production of complete recombinant or chimeric gonadotropins. Various procedures for producing recombinant GTHs have utilized both prokaryotic and eukaryotic cell systems (Shoham and Insler 1996). In order to produce active recombinant GTHs, the structural characteristics of the protein needs to be considered. Activity of the GTH hormones is based on several specific physical properties and post-translational modifications. The protein must be folded properly, including the presence of the cysteine knot that is representative of all gonadotropin hormones. At three sites in the protein, the cysteine residues form a disulfide bridge and provide the molecular tertiary structure. The glycoprotein itself exists as two subunits (α and β) and is associated by noncovalent bonds to form a heterodimer (Combarnous 1992). The α subunit is common for all gonadotropins of a given species and is necessary for proper function. Hormone activity has been directly linked to the formation of a functional heterodimer, even though the hormone specificity relies on the low percent identity of each beta subunit to other glycoproteins (Vischer et al., 2003). Therefore, the strategy for producing recombinant GTH was to produce a heterodimer protein. Since the alpha subunit of GTH has yet to be determined in lampreys, other subunits from other species were tested for heterodimer interaction.

Due to the extensive post-translational glycosylation patterns of gonadotropins, mammalian cell lines have been the most effective in producing a functional hormone (Shoham and Insler 1996). For small scale production of recombinant protein, Chinese Hamster Ovary (CHO) cells were used to isolate biologically active bovine LH (Kaetzel et al., 1985). On a large scale, the SF-9 army worm cell line was combined with the baculovirus infection system to produce functional recombinant human chorionic gonadotropin (hCG) with similar glycosylated moieties (Chen et al., 1991). The *Drosophila melanogaster* cell line has been tested and shown to produce large amounts of intact recombinant channel catfish FSH and LH at levels exceeding 6 mg/L (Zmora et al., 2003). Following protein production, the purification of the recombinant must be done. Purification can be attained using simple affinity chromatography against the glycosylated proteins using Concanavalin A, or through protein His-tagging and purification with Nickel Sepharose. Using transfected cell lines and the Drosophila Expression System (Invitrogen, 2004) along with stably transfected CHO cell lines, the purpose of this study was to produce a recombinant heterodimer lamprey GTH with essential glycosylation and a hexahistidinyl moiety attached to the C-terminal end.

MATERIALS AND METHODS

Animals

Reproductively mature male and female sea lamprey were collected from Cocheco River fish ladder in Dover, NH, USA during their upstream migration in May from 2004 to 2007. They were transported to the Anadromous Fish and Aquatic Invertebrate Research (AFAIR) Laboratory at the University of New Hampshire. The lamprey were maintained under continuous flow of river water and aeration at ambient temperature ranging from 14-18°C. Following decapitation and dissection, sea lamprey tissue samples were collected at the UNH AFAIR laboratory.

Cell Culture Maintenance

Chinese Hamster Ovary (CHO) cells were obtained from American Type Culture Collection at low passage and grown in T25 flasks containing 5 mLs of RPMI-1640 supplemented with phenol red, L-Glutamine, 25 mM Hepes, sodium bicarbonate, and 10% FBS. Cells were cultured at 37°C and 5% CO_2 in a humidity controlled, water jacketed incubator (Forma Scientific 3110). After two weeks and three passages, frozen stocks were prepared of wild type cell lines. Cells were passaged when the cultures reached 95% confluency, and seeded at approximately 100,000 cells/mL in RPMI-1640 growth medium.

Drosophila melanogaster (S2) cells were purchased through Invitrogen as part of the Drosophila Expression System (DES). Cells were thawed and transferred into 4 mLs modified Schneider's insect medium w/ 10% heat denatured FBS (30 min at 65°C) and

1% Penicillin/Streptomycin (p/s), and incubated at room temperature (20-25°C). Cells were grown in suspension in T25 flasks up to confluency ($1X10^7$-$2X10^7$ cells/ml) where they were passaged into a new T25 or larger T75 flask. During passaging, conditioned medium was transferred with the cells into the new flask containing fresh growth medium (Schneider's, 10% heat denatured FBS, 1% p/s). Frozen stocks were prepared from wild type cell lines before transfection.

Plasmid Preparation

The lamprey GTH-β coding sequence was received from Dr. Shunsuke Moriyama (Kitasato University School of Fisheries Sciences, Sanriku Iwate). The cDNA was amplified and inserted into the pGCAP1 vector. The vector was amplified using designed forward (5'-ATAGGTACCACCATGGGTCCCCTTCAGCTGTTCCAAC-3') and reverse (5'-ATAACCGGTAACCCGGTAGCTGCCCGCAGCTG-3') primers against the GTH-β sequence (Table 1) under the following cycling conditions: (1) initial denaturation 2 min/95°C, (2) 5 cycles with denaturation 40 s/94°C, annealing 30 s/70°C, extension 1 min/72°C, (3) 5 cycles with denaturation 40 s/94°C, annealing 30 s/68°C, extension 1 min/72°C, (4) 5 cycles with denaturation 40 s/94°C, annealing 30 s/66°C, extension 1 min/72°C, and (5) 30 cycles with 40 s/94°C denaturation, 30 s/64°C annealing, 1 min/72°C extension. The desired PCR product was then isolated from 2% agarose gel using the QIAEX-II gel extraction kit (Qiagen Inc.) and digested to completion with 15U of KpN-I and Age-I restriction enzymes (Promega Corp.). The sequence was then ligated into linearized pMT/V5-His expression vector, received as a gift from Dr. John Trant (UMBI, Baltimore, Maryland) from the DES system

(Invitrogen) using 1.5 U of T4 ligase. Ligated vector product was transformed into competent JM109 cells (Promega Corp.) and bacterial plasmid was isolated from 3 mLs LB culture medium using Wizard plus Mini-prep (Promega Corp.).

Primer Name	**Nucleotide Sequence (5'-3')**
lGTHb5_1	ATA**GGTACC**ACCATGGGTCCCCTTCAGCTGTTCCAAC
lGTHb3_1	ATA**ACCGGTA**ACCCGGTAGCTGCCCGCAGCTG

Table 1: These primers were used for the initial amplification of lamprey GTH-β from plasmid and preparation of the lGTH-β S2 and CHO expression constructs.

Preparation of Tethered Expression Constructs

Primer names and sequences for the development of tethered constructs are listed in table 2. PCR reactions for all the constructs used the Phusion high fidelity DNA polymerase (Finnzymes, New England Biolabs). Plasmid containing the lamprey GTH-β construct was amplified with new 3' primers (lGTHbeta R1) to insert BamH-I restriction sites for the addition of a 15 amino acid serine/glycine linker and one of two alpha subunits; ccGTH-α (channel catfish), or hCG-α (human). Channel catfish 5' (ccGpHa_link_for) and 3' (ccGTHa3_1) primers were used to isolate an amplified sequence from pMT/V5-His plasmid containing the intact alpha subunit (John Trant; Baltimore, Maryland), adding the serine/glycine linker along with BamH-I and Age-I restriction sites at the 5' and 3' ends respectively. Human CG alpha (Dr. Bill Moyle, Piscataway, NJ) was amplified from plasmid with 5' (hGPHalpha F2L) and 3' (hGPHalpha R1), inserting the same restriction sites and protein linker as used in the amplification of ccGTH-α. Amplified lGTH-β, hCG-α, and ccGTH-α were restricted with KpN-I, BamH-I, and Age-I enzymes and ligated together to form the following constructs: lGTH-β/hCG-α (6XHis); lGTH-β/hCG-α (no His); lGTH-β/ccGTH-α (6XHis); lGTH-β/ccGTH-α (no His). A schematic diagram showing the ligation of lGTHB/hCG-a (His) into pMT/V5-His vector is shown in Figure 8.

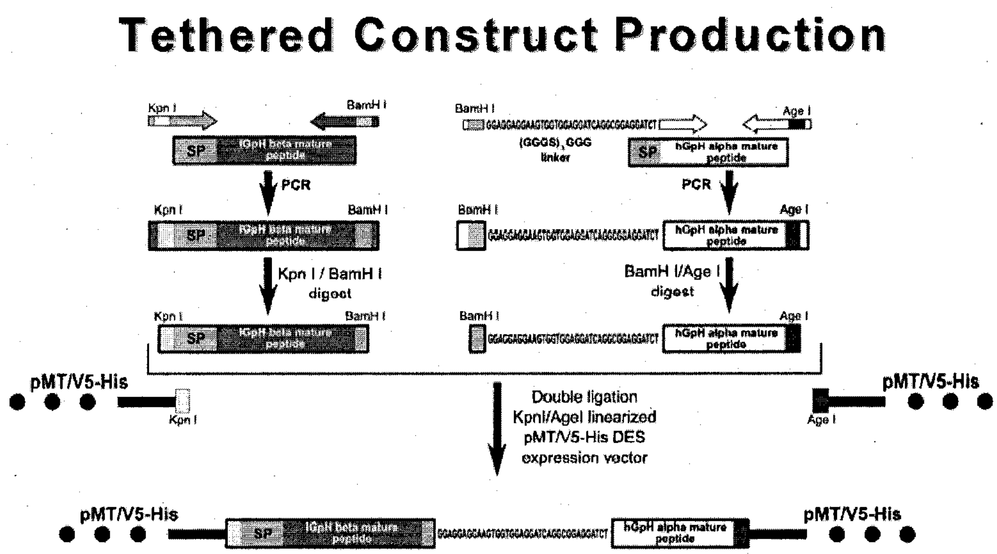


Figure 8: A schematic diagram showing the PCR primers and restriction enzymes necessary to combine lGTH-B with hCG-a, which includes a serine/glycine linker (Freamat, 2007).

Two-step overlapping PCR was used in order to allow for the addition of a N-terminal 6XHis tag into the lGTH-β/hCG-α tethered construct. Sequences were amplified through PCR, restricted, then inserted into either the pMTV5His (Insect cell expression) or pCDNA3.1-V5His (mammalian expression) plasmid. Step 1 involved the amplification of two separate products from the tethered lGTH-B/hCG-a construct. Using 5' (MT sequencing primer) and 3' (lGpHb_SP_his_r) in one reaction and 5' (lGpHb_his_MP_f) and 3' (BGH sequencing primer) in a separate reaction, two PCR fragments containing overlapping 6XHis tags were produced. In step 2, both amplified fragments were combined into one PCR reaction, and amplified using 5' (MT sequencing primer) and 3' (hGpHalpha R1) to yield one amplified product with a 6XHis tag at the N-terminus of lGTH-β. The amplified product was then isolated from agarose gel (Qiaex II, Qiagen) and restricted with KpN-I and Age-I enzymes to allow for insertion back into the pMT/V5-His expression vector. The same amplified construct was later ligated into the pCDNA3.1-V5His (mammalian) expression vector for CHO cell expression.

Primer Name	Target	Sequence (5'-3')	Modification
lGTHβ(His)			
lGTHb5_1	lGTHB	ATAGGTACCACCATGGGTCCCCTTCAGCTGTTCCAAC	KpN-I and Kozak
lGTHb3_1	lGTHB	ATAACCGGTAACCCGGTAGCTGCCCGCAGCTG	Age-I and 6XHis
ccGTHα			
CcGTHa5_1	ccGTH a	ATAGGTACC ACCCACCATGATTCTGATTCTTAAATAC	KpN-I and Kozak
CcGTHa5_2	ccGTH a	ATAGGTACCACCCACCATGATTCTGATTCTTAAAT	KpN-I and Kozak
ccGTHa3_1His	ccGTH a	ATAACCGGTAAATTTATGATAGTAACAAGT	Age-I/6xHis
ccGTHa3_1	ccGTH a	ATAACCGGTCTAAAATTTATGATAGTAACAAGT	Age-I/No His
hGPHα			
hGpHalpha F1	hGpH a	ATAGGTACCACCATGGATTACTACAGAAAATATGCAGC	KpN-I and Kozak
hGpH alpha R1	hGpH a	ATAACCGGTTTAAGATTTGTGATAATAACAAGT	Age-I/STOP (no 6XHis)
hGpHalpha R2	hGpH a	ATAACCGGTAGATTTGTGATAATAACAAGTACTGC	Age-I/no STOP (6XHis)
lGTHβ/ccGTHα (C-term His)			
lGTHb5_1	lGTHB	ATAGGTACCACCATGGGTCCCCTTCAGCTGTTCCAAC	KpN-I and Kozak
lGTHbeta R1	lGTHB	ATAGGATCCAACCCGGTAGCTGCCCGCAGCTG	BamH-I/no STOP
ccGpHa_link_for	ccGTH a	ATAGGATCCGGAGGAGGAAGTGGTGGAGGATCAGGCGGAGGAT CTGGAGGAGGATTTCCAAACAATGACTTTGGCTG	BamH-I/Tether
ccGTHa3_1His	ccGTH a	ATAACCGGTAAATTTATGATAGTAACAAGT	Age-I/6xHis
ccGTHa3_1	ccGTH a	ATAACCGGTCTAAAATTTATGATAGTAACAAGT	Age-I/No His
lGTHβ/hGPHα (C-term His)			
lGTHb5_1	lGTHB	ATAGGTACCACCATGGGTCCCCTTCAGCTGTTCCAAC	KpN-I and Kozak
lGTHbeta R1	lGTHB	ATAGGATCCAACCCGGTAGCTGCCCGCAGCTG	BamH-I/no STOP
hGpHalpha F2L	hGpH a	ATAGGATCCGGAGGAGGAAGTGGTGGAGGATCAGGCGGAGGAT CTGGAGGAGGAGCTCCTGATGTGCAGGATTG	BamH-I/Tether
hGpH alpha R1	hGpH a	ATAACCGGTTTAAGATTTGTGATAATAACAAGT	Age-I/STOP (no 6XHis)
hGpHalpha R2	hGpH a	ATAACCGGTAGATTTGTGATAATAACAAGTACTGC	Age-I/no STOP (6XHis)
lGTHβ/hGPHα (N-term His)			
MT	pMTV5His	CATCTCAGTGCAACTAAA	Seq. primer (forward)
lGpHb_SP_his_r	lGTHB	ATGGTGGTGATGATGGTGGGAGTAAGCCACCTCGAGCC	His tag/ partial SP
BGH	pMTV5His	TAGAAGGCACAGTCGAGG	Seq. primer (reverse)
lGpHb_his_MP_f	lGTHB	CACCATCATCACCACCATAACTCCTTGTGCAAGCTGCA	His tag/ partial MP
lGpHb_KpnI_SP_f	lGTHB	ATAGGTACCACCATGGGTCCCCTTCAGCTGTTCCAAC	KpN-I/ partial SP
hGpH alpha R1	hGpH a	ATAACCGGTTTAAGATTTGTGATAATAACAAGT	Age-I/STOP (no 6XHis)

Table 2: A comprehensive list of all primers designed for the preparation of tethered gonadotropins, as well as for co-transfection of ccGTH-α and hCG-α subunits.

Transfection of S2 cells

Transfection procedures followed those described by Trant et al. 2003. Initial transfection of the S2 cells utilized the Effectene Reagent (Qiagen) to co-transfect the pCoBlast selection vector with the pMT-V5-His plasmid containing the lamprey GTH-β insert into S2 cells. Using the DES expression system, a stable line was developed using Blasticidin antibiotic (25 μg/ml) selection over a period of four weeks. Two other cultures were also transfected and selected for a 4 week period: lGTH-β with ccGTH-α; green flourescence protein (GFP) transfection efficiency control. The GFP transfected cells were tracked during selection using UV detection to estimate the number of cells expressing protein. The gonadotropin transfected cultures were expanded into 1 L shaker flasks in growth medium and brought to confluency ($2X10^7$ cells/ml) as described by the manufacturer's protocol (DES System, Invitrogen). Cells were centrifuged in sterile (4) 250 ml bottles at 1,000Xg for 15 minutes. Medium was replaced with serum free Schneider's and induced for 24-48 hours with Copper Sulfate (500 μM). Culture medium was centrifuged at 1000Xg for 30 minutes, and then filter sterilized and collected for further purification. Cells were lysed and stored at -80°C to check for intercellular protein and expression.

The Effectene reagent was not used in the second round of transfection, instead the calcium phosphate reagent was utilized as described by the DES kit. Cells were transfected with combinations of constructs along with the selection plasmid (pCoBlast): blank (selection control); lGTH-β and ccGTH-α co-transfection; lGTH-β and ccGTH-α (His) co-transfection; tethered lGTH-β/ccGTH-α (C-term His); tethered lGTH-β/hCG-α (C-term His); tethered lGTH-β/hCG-α (N-term His); lGTH-β and hCG-α co-transfection.

All transfections occurred in a 24-well plate with 600,000 cells/ 500 μLs serum free medium. The cells were transferred into growth medium 24 hours post transfection and incubated at room temperature. After 48 hours, 25 μg/ml Blasticidin was added to each culture, which remained under constant selection for a period of 4 weeks. Growth, expansion, and induction of cells using copper sulfate followed the same protocol as described above.

Transfection of CHO Cells

Using the Lipofectamine 2000 transfection reagent (Invitrogen, 2008), confluent CHO cells were transfected according to manufacturer's protocol in T-25 flasks with 10 μg of the following pCDNA3.1/V5-His constructs: lGTH-β; cotransfected lGTH-β and ccGTH-α, cotransfected lGTH-β and hCG-α; tethered lGTH-β/ccGTH-α; tethered lGTH-β/hCG-α (N-term. His); tethered lGTH-β/hCG-α (C-term. His); tethered lGTH-β/hCG-α (No His). Transfected cultures were transferred into fresh growth medium after 24 hours, and after 48 hours were stably selected using 1 mg/ml G418 antibiotic over a period of 2 weeks. Constitutively expressing cell lines were clonally selected using the 96-well plate isolation method. Only 2-3 isolated cultures were expanded and used for further studies. The cells were trypsinized, centrifuged at 1,000Xg for 10 minutes, and lysed to check for recombinant protein expression using RT-PCR. CHO cells were maintained under continuous selection during expansion of cell lines.

RT-PCR

S2 and CHO cells from each of the stable transfected cultures ($5X10^6$ cells) were centrifuged at 1000Xg, and extracted for total RNA using MRC Tri-Reagent (Molecular Research Center, 2005) protocol. Approximately 40 μg of total RNA was isolated from each sample. 3 μg of total RNA was diluted to 5 μl with milli-Q water plus PCR buffer and treated with 3 μl Promega RQ-1 DNase for 2 hours at 37°C. The sample was analyzed through AccessQuick RT-PCR kit (Promega, 2007) using the gene specific forward and reverse primers for rlGTH-β. Oligonucleotide primers lGTHbGSP1_r (5'-GTTTCGTTGGTCAGATCGACTCCAAGC-3') and lGTHbGSP1_f (5'-GAGTGTCGTTACATCAACACCACCGTC-3') were utilized at 2 μM in each reaction mixture of 25.5 μl (including 1 μl template, 0.5 μl reverse transcriptase). Preliminary incubation occurred for 45 minutes at 45°C for reverse transcription. Subsequent PCR involved 35 cycles of amplification. After activation of *Taq* at 95°C for 2 minutes, each cycle consisted of 40 sec denaturation at 94°C, 30 sec primer annealing at 70°C, and 1 minute primer extension at 72°C. Results were compared against a positive control of prepared plasmid lGTH-β as a template and a negative control with no template, as well as S2 RNA template without reverse transcriptase. The final PCR amplified product was electrophoresed on 2% agarose gels and visualized using ethidium bromide staining (Nippon Gene).

Production and Purification from CHO cell lines

Small scale cultures from mammalian CHO cells were expanded into larger T-75 flasks and frozen stocks of $1X10^6$ cells/ml were prepared after verification of lGTH-β expression using RT-PCR. Frozen stocks were transferred to NHCTC (New Hampshire Community Technical College, Portsmouth, NH) for further growth and expansion at their facility. These adherent cultures were adapted for suspension in 50 mL Bellco Spinner bottles at 37°C and 5% CO_2, then transferred to larger 1-3 L bioreactors (New Brunswick Bioflo 3000) under continuous selection with G418 sulfate in RPMI-1640, 10% FBS at 37°C and 5% CO_2.

Cells were transferred to reduced serum medium containing RPMI-1640, 1% FBS before final collection. Cells were grown for 24-48 hours before collection of growth medium. Cells were removed through centrifugation and growth medium was then filtered using TFF ultrafiltration (Pall, 2007) at 100K MWCO. The eluant was then concentrated using TFF ultrafiltration at 10K MWCO. A small portion of the final product was probed using Western Blot to check for isolated recombinant lamprey gonadotropin. Protein was detected using the Pierce Supersignal West-Pico 6XHis detection kit (Pierce, 2004). Other than the 6XHis probe, the Western was also probed with rabbit anti-hCG antibody (Biospacific, #R-114-C), and an antibody produced against a 16 amino acid partial peptide of lGTH-β at positions 52-68 (YTWQLIGHNMRKIAQEV), which had been used previously to detect lGTH-β immunohistochemically (Sower et al., 2006). Purified protein samples were also used for *in vitro* lamprey gonadal culture and COS-7 cAMP receptor activity assays.

Production and purification from S2 cell lines

Medium from insect cell cultures was partially purified through the isolation of all histidine rich proteins using Nickel Sepharose affinity chromatography. For each liter of medium, approximately 10 mls of Ni-Sepharose were necessary to bind the total protein in a buffered solution containing 20 mM PBS, 500 mM NaCl, and 20 mM Imidazole. The column was rinsed with 4 column volumes of binding buffer at a flow rate of 2.5 ml/min using a Biorad Flow Adapter. The column was eluted in 1 mL fractions with 20 mM PBS, 500 mM NaCl, and 500 mM Imidazole and then each fraction was analyzed using SDS-PAGE and the Supersignal West Hisprobe Kit following the manufacturers protocol (Pierce). Protein was TCA precipitated before SDS-PAGE analysis using 30 μl of cold 100% TCA and 200 μl of each fraction. Fractions containing protein of interest were pooled and concentrated using centrifugation at 3,000Xg with the Amicon Centricon Plus-20 (10,000 MWCO). The concentration was followed by buffer exchange using subsequent centrifugation and 1X PBS. A Bradford assay (Pierce, 2006) gave estimates of total concentrated protein collected from each of the purifications. Concentrated partially purified samples were then used to test for protein activity through *in vitro* gonadal culture and *in vitro* cAMP response.

In Vitro Culture

Samples purified from S2 cell cultures were concentrated and tested using *in vitro* culture of lamprey gonads as previously described (Gazourian et al., 1997). Concentrated medium from CHO cells was also tested. Gonadal tissues were collected from adult male and female sea run lampreys. Tissues were cut up into small pieces (males ~40 mg, females ~15 mg) and divided into a 24-well plate containing 500 μl Hanks Balanced Salt Solution (HBSS) with 25 mM Hepes (pH 7.0). Tissues were pre-incubated for 2 hours at 18°C with no treatment. Pre-incubation medium was collected and ovaries and testes were incubated in triplicate with each of three treatments: Saline Control, purified rlGTH-β, and purified SG-G100. SG-G100 is a partially purified salmon gonadotropin prepared through ethanol extractions and gel filtration techniques at the Department of Fisheries and Oceans, West Vancouver Laboratory, Canada (Sower et al., 1982). Each hormone was introduced at concentrations of 10, 100, 1000 ng/mL. Pre-incubation and incubation media was collected at 2 hours and 18 hours respectively. Media estradiol concentrations were measured by radioimmunoassay as described previously (Sower et al., 1983; Sower and Schreck 1982).

Receptor activity using cAMP EIA

Concentrated medium from stably selected CHO cells including purified protein from large scale recombinant protein production were tested for activity using the Biotrak EIA (enzyme-linked immunoassay) for intracellular cAMP response (RPN225, GE HealthCare LifeSciences, 2008). COS-7 cells, grown at 37°C and 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM) with 10% FBS, were transfected according to manufacturers protocol in 60 millimeter plates using Lipofectamine 2000 (Invitrogen, 2007) with constructs containing either lamprey GPH-R I (Freamat et al., 2006), Rat LH-R (Provided by Dr. William R Moyle, 2006), or blank pCDNA3.1 (negative control) vector construct following the procedure outlined in previous studies (Zmora et al., 2003). Cells were re-plated after 24 hours into 96 well plates to obtain 100,000 cells/well (100μl/well). After 48 hours, the cells were subjected to serum starvation with DMEM and 0.5% FBS. Cells were incubated overnight in the incubator. Transfected cells were treated with the following medium samples and treatments in duplicate: wild type CHO medium, CHO purified lGTH-β/hCG-α (N-term 6XHis), CHO purified lGTH- β/hCG-α (no 6XHis), CHO purified lGTH-β/hCG-α (C-term 6XHis), S2 nickel sepharose purified lGTH-β/hCG-α (C-term 6XHis), 5 μM Forskolin (maximal stimulation), and basal medium. All incubation samples were prepared in basal medium consisting of DMEM, 1 mg/ml BSA, and 0.2 mM IBMX (3-Isobutylmethylxanthine, limits degradation of cAMP). Cells were lysed and the intracellular cAMP accumulation was measured using enzyme-linked immunoassay according to the manufacturer's instructions (GE Lifesciences, 2008).

Statistics

Analyses for the cAMP and estradiol assay data was performed with one-way ANOVA using Excel (Microsoft, 2008) and Systat (Systat Software Inc, 2008). Results from one way ANOVA were then compared for significance using Dunnett's test against the control group.

RESULTS

Plasmid Preparation

The amplification and ligation of constructs for lGTH-β, ccGTH-α, hCG-α, and variations with or without 6XHis tags were successful with the use of properly designed primers. Full length clones of all three subunits were easily inserted into pMT/V5-His and pCDNA3.1/V5-His expression vectors, and the insert size matched what was expected. Tethered lGTH-β/hCG-α consisted of approximately 800 bp, including the 15 amino acid linker to fuse the two subunits. All amplified clones were sequenced (University of Utah Sequencing, Salt Lake City, UT) to verify proper insertion, and were found not to contain any mutations that could possibly alter protein function. Figure 9 shows a plasmid digestion with KpN-I and Age-I on an agarose gel to verify the presence of each of the following inserts: lGTH-β, hCG-α, tethered lGTH-β/hCG-α, ccGTH-α, ccGTH-α (His).

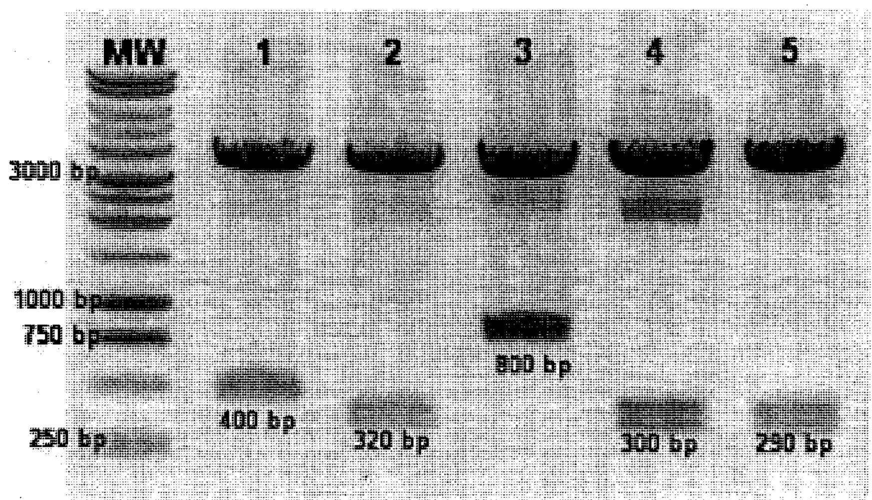


Figure 9: Restriction digest w/ KpN-I and Age-I enzymes to verify presence of inserts and proper preparation of tethered construct: Lane 1 (lGTH-β), lane 2 (hCG-α), lane 3 (tethered lGTH-β/hCG-α), lane 4 (ccGTH-α), lane 5 (ccGTH-α His)

Preparation of Tethered Expression Constructs

Tethered constructs that were prepared in both pMT/V5-His and pCDNA3.1/V5-His were successfully prepared using a combination of standard PCR protocols and overlapping two-step PCR. Three tethered constructs were prepared in pCDNA3.1/V5-His for transfection into CHO cells: lGTH-β/hCG-α (N-term 6XHis), lGTH-β/hCG-α (no 6Xhis), lGTH-β/hCG-α (C-term 6XHis). For each Wizard-Plus Miniprep (Promega), approximately 10 μg of plasmid was isolated from transformed JM-109 and TOP-10 competent bacterial stocks.

Transfection of S2 cells

Although there were several failed attempts at developing stable S2 cells, one technique was most effective at properly transporting the DNA into the cell. Using Lipofectamine 2000 for S2 cell lines was ineffective, and stable cell lines were not successfully achieved after 4 attempts. The use of a second liposomal reagent, Cellfectin (designed for mammalian and insect cell transfection, Invitrogen), also did not produce stable cell lines. Using the Calcium Phosphate coprecipitation method, available with the DES kit, led to repetitive stable S2 cell lines. The cell mortality post transfection was low, but many of the cell lines had high levels of cell death during the start of selection with Blasticidin. All cell lines that were selected with Blasticidin (25 μg/ml) recovered after one week of selection, and began showing signs of increased growth after two weeks.

Transfection of CHO cells

Each of the stable CHO cell lines was transfected using Lipofectamine 2000, which successfully yielded 5 stable lines. From the Lipofectamine 2000 transfections, the following stable cell lines were produced: lGTH-β, cotransfected lGTH-β and hCG-α, tethered lGTH-β/hCG-α (C-term His), tethered lGTH-β/hCG-α (no His), tethered lGTH-β/hCG-α (N-term His).

RT-PCR

Many attempts to express the lGTH-β protein successfully in both CHO and S2 cells were unsuccessful. From 10 different stable S2 insect cell lines developed, only the following five were successfully shown to express the lGTH-β signal through Accessquick RT-PCR: lGTH-β, lGTH-β and ccGTH-α coexpression, lGTH-β/ccGTH-α(His) coexpression, tethered lGTH-β/hCG-α, lGTH-β and hCG-α coexpression. Each culture was successfully determined to be expressing the GTH-β transcript (310 bp), as shown (Figure 10, A).

Three cell lines from CHO stable transfections were determined to positively produce the lGTH-β signal through RT-PCR. Earlier attempts had been made to isolate stable cell lines from CHO, but the proper lGTH-β signal could not be identified by RT-PCR. The following cell lines were successfully producing the signal: lGTH-β/hCG-α (N-term 6XHis), lGTH-β/hCG-α (no 6Xhis), lGTH-β/hCG-α (C-term 6XHis) (Figure 10, B).

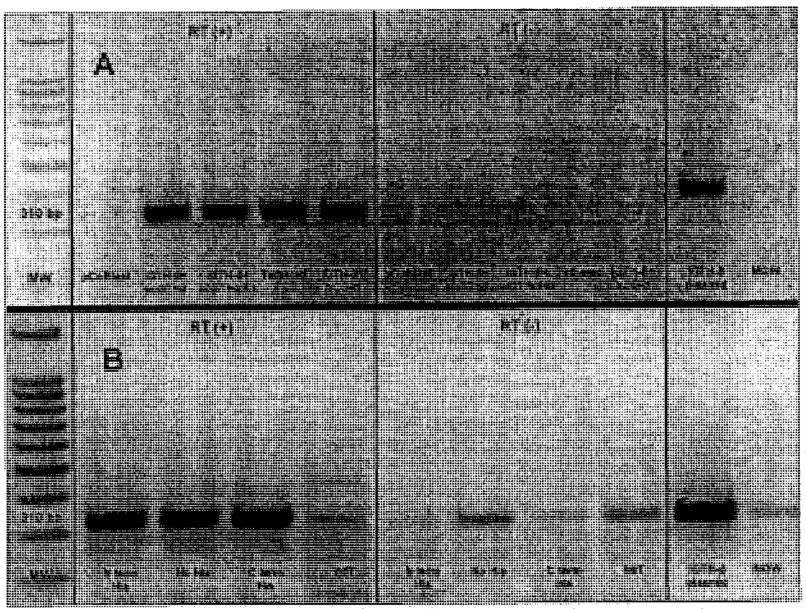


Figure 10: Results from Accessquick RT-PCR (Promega, 2007) indicated that the lamprey GTH-β transcript was expressed in both S2 cell (A) and CHO cell (B) stable lines. Reverse Transcriptase was added to the first set of reactions in both gels, and was not added in the second set of reactions. lGTH-β construct in pMTV5His plasmid was used as a positive control.

Production and Purification from S2 cells

Histidine tagged recombinant proteins produced from induced S2 cell lines were successfully identified by western blot. Using the Pierce HisProbe, concentrated medium from expressing S2 cell lines were probed before and after induction with copper sulfate. After induction the media showed the presence of recombinant gonadotropins at high levels. An example of the S2 cell induced recombinant gonadotropin beta subunit protein expression is shown in Figure 11. Secreted protein product from S2 cells was only detectable on western blot in the lGTH-β individually transfected cell line, and could not be detected from the co-expressed stable lGTH-β with ccGTH-α.

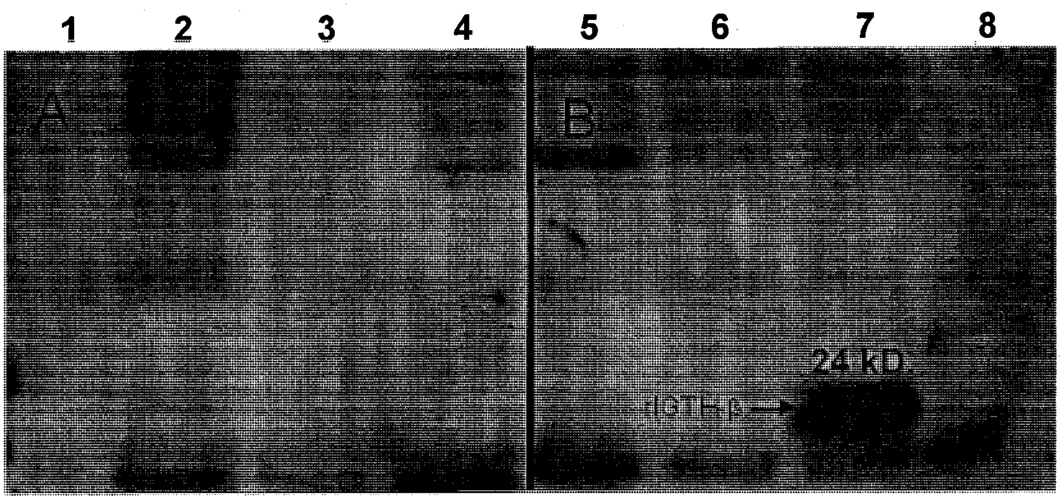


Figure 11: A western blot showing the non-induced (A) vs. induced (B) S2 cell cultures for 3 of the transfections, including wild type: lanes 1 and 5 (w/t), lanes 2 and 6 (ccGTHα), lanes 3 and 7 (lGTH-β), lanes 4 and 8 (lGTH-β and ccGTHα cotransfection). Successful secreted protein production was only detectable in the lGTH-β cell line (approx. 16 kD.).

From approximately 10 large scale nickel sepharose purifications from 1L S2 cell cultures, detectable protein could be isolated from 4 of the elutions. Optimization of the elution profile and binding conditions of the affinity beads yielded a repeatable elution from concentrated S2 culture medium. Western Blot verified the successful binding and elution of protein from the column (Figure 12). Elution of purified S2 culture protein from nickel sepharose was separated into five 1 ml fractions and pooled into a total of 100 μl medium concentrate. The total amount of protein yielded from each nickel sepharose elution was approximately 200 μg.

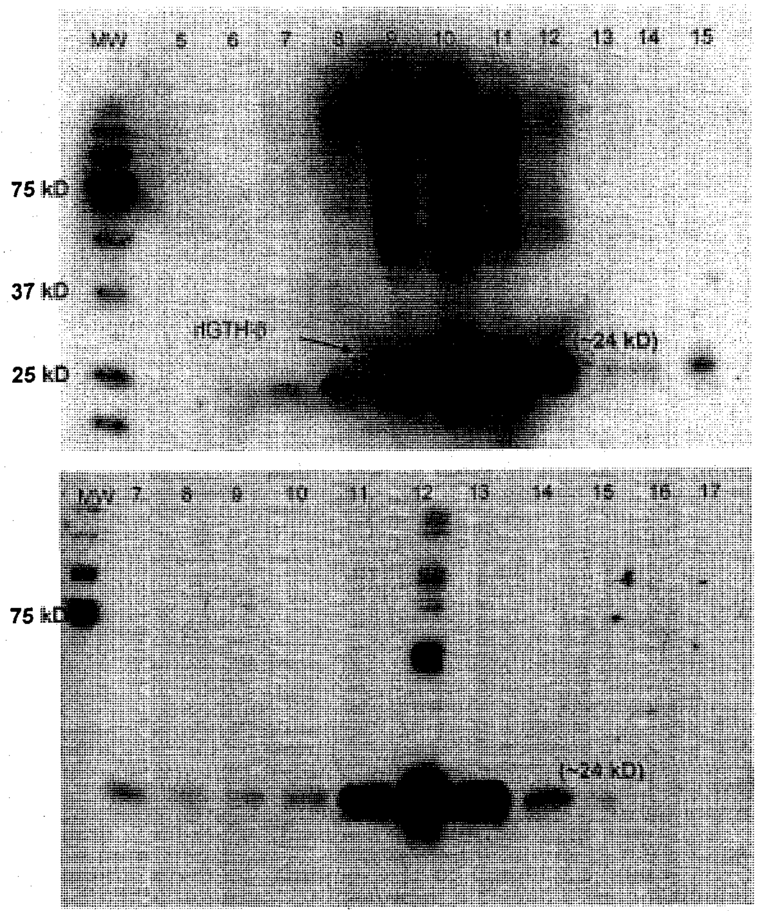


Figure 12: Optimization of Nickel Sepharose purification and the removal of FBS from induction medium (2nd Western) in S2 cells yielded a much cleaner elution of lGTH-β recombinant protein, with minimal background. Bound protein was eluted with 500 mM Imidazole in PBS (50 mM NaH_2PO_4, 500 mM NaCl). A total of 200 μg protein was eluted and concentrated from each run of nickel sepharose.

Production and Purification from CHO cell lines

Approximately 100 mls of final concentrated protein product was isolated from 3 liters of growth medium using TFF ultrafiltration with 10K and 100K MWCO. Attempts to purify His-tagged protein product from stable CHO cell lines using nickel sepharose were unsuccessful from all three tethered gonadotropin stable cell lines. The presence of eluted protein from the column, as well as definitive evidence of a highly produced tethered gonadotropin were not detected using western blot. Further concentration of 100 mls of medium used 10K MWCO centrifugal concentrators, to a final volume of 200 μl. Figure 13 shows the western blots that were used to identify recombinant protein in the medium, probed with either a 6XHis-HRP probe, lamprey GTH-β antibody, or hCG-α antibody. Results from 6XHis probe and hCG-α western were positive for the Nickel sepharose purified S2 product (lane 9, figure 13), but were inconclusive with the lamprey GTH-β antibody. The background could not be eliminated from the lGTH-β western, and therefore could not be used to show the production of gonadotropins from CHO cells. Even more importantly, there was no detectable gonadotropin from any of the CHO cell medium samples using two highly specific antibodies (6XHis-HRP and hCG-α). The final concentrate was used for *in vitro* culture and cAMP accumulation assays in order to test for any protein activity.

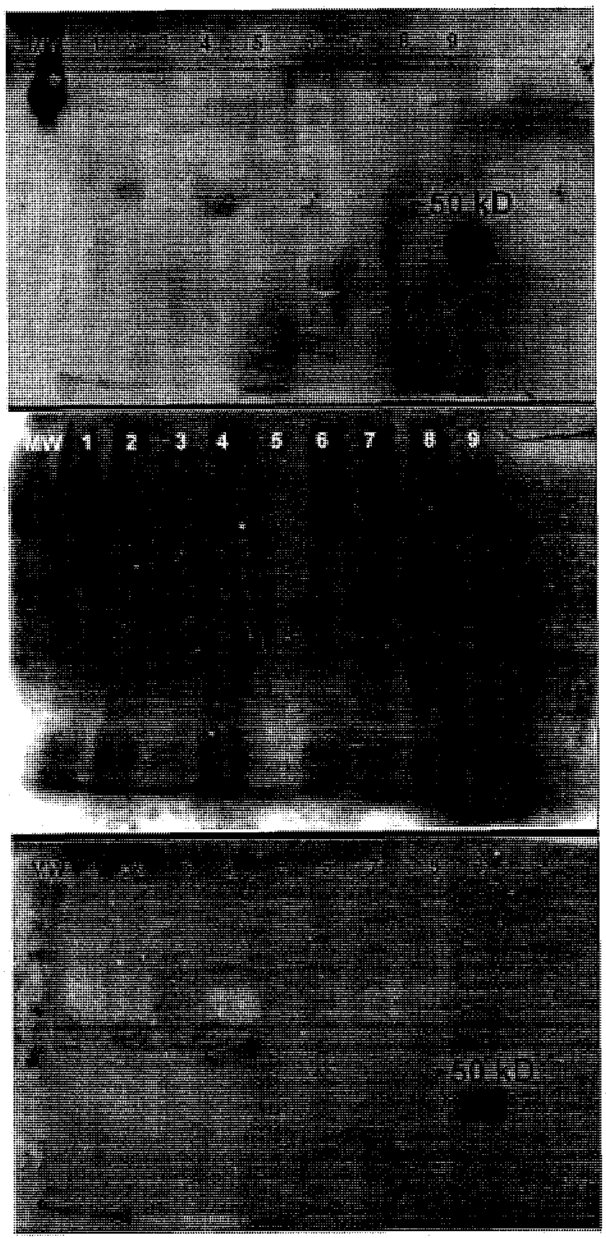
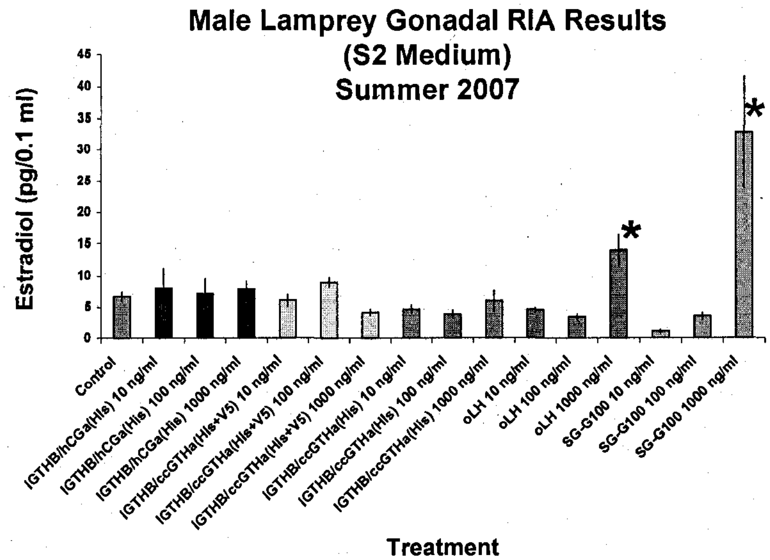


Figure 13: Results from three western blots probed with either (1) 6XHis-HRP probe (Pierce, 2005), (2) lamprey GTH-β Ab (Sower et al., 2006), and (3) hCG-α Ab (Biospacific, #R-114-C). The lGTH-β antibody and hCG-α antibody both used 2° Goat anti-rabbit IgG-HRP (Thermo scientific, # 31463). Samples from left to right: 1, 2- CHO stable medium lGTH-β/hCG-α (N-term His), 3- CHO stable medium lGTH-β/hCG-α (No His), 4- CHO stable medium lGTH-β/hCG-α (C-term His), 5- Wild type CHO medium, 6- transient CHO lGTH-β/hCG-α (N-term His), 7- transient CHO lGTH-β/hCG-α (No His), 8- CHO stable medium lGTH-β/hCG-α (C-term His), 9- Ni-seph purified lGTH-β/hCG-α (S2 cells).

In Vitro Culture- Testing purified S2 medium

Purified samples from S2 cell cultures did not elicit a positive response from male or female lamprey gonadal tissue as determined by analysis of the estradiol data. Gonadal samples incubated with concentrated tethered lGTH-β/hCG-α medium did not have a higher concentration of estradiol (8±2.4 pg/0.1 ml), compared to those samples incubated with SG-G100 positive control (33±8.8 pg/0.1 ml). Other treatments of concentrated culture medium did not show a response higher than control (6.7±0.8 pg/0.1 ml). Female lamprey gonadal tissue did not respond significantly ($P>0.05$) to any of the concentrated S2 medium samples, and did not have a significant positive response to SG-G100 control (Figure 14).



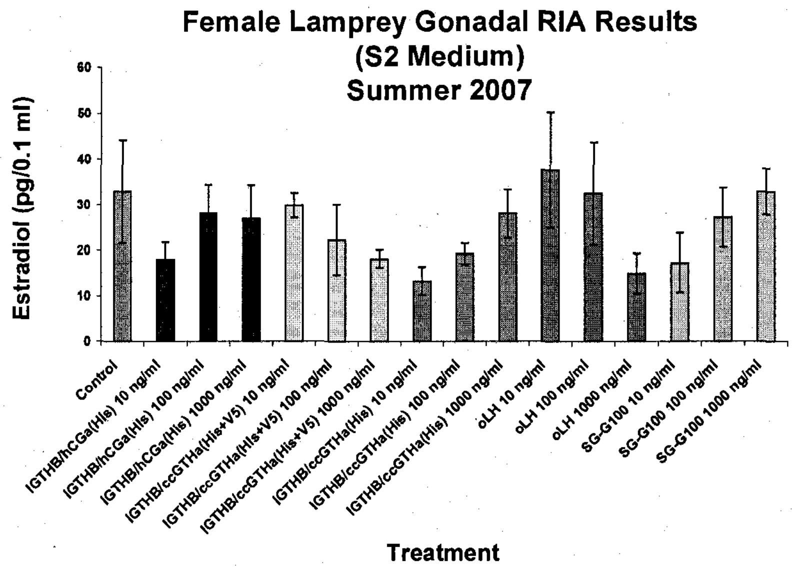


Figure 14: The response of lamprey male and female gonadal tissue to recombinant lamprey gonadotropins isolated from S2 cell culture medium was measured in pg estradiol/0.1 ml medium using radioimmunoassay. Even though the response to SGG100 (partially purified salmon GTH) in males was 5 fold higher than control ($P<0.05$), there was no significant response from any of the purified S2 gonadotropin samples when compared to the control. Ovine LH did show a significant response in males ($P<0.05$).

In-vitro culture- Testing purified CHO medium

Before large scale production of stable CHO cell lines, media from G418 selected cell lines were concentrated and tested using in-vitro culture. Results from in-vitro gonadal culture (Figure 15) did indicate an estradiol response to concentrated medium from CHO cells transfected with tethered lGTHβ/hCGα construct (396±86 pg/0.1 ml) that was 3-fold greater than the HBSS control (107±16 pg/0.1 ml) ($P<0.05$). The response of CHO medium from tethered constructs was also higher than the SG-G100 positive control incubations (274±38 pg/0.1 ml) ($P>0.05$).

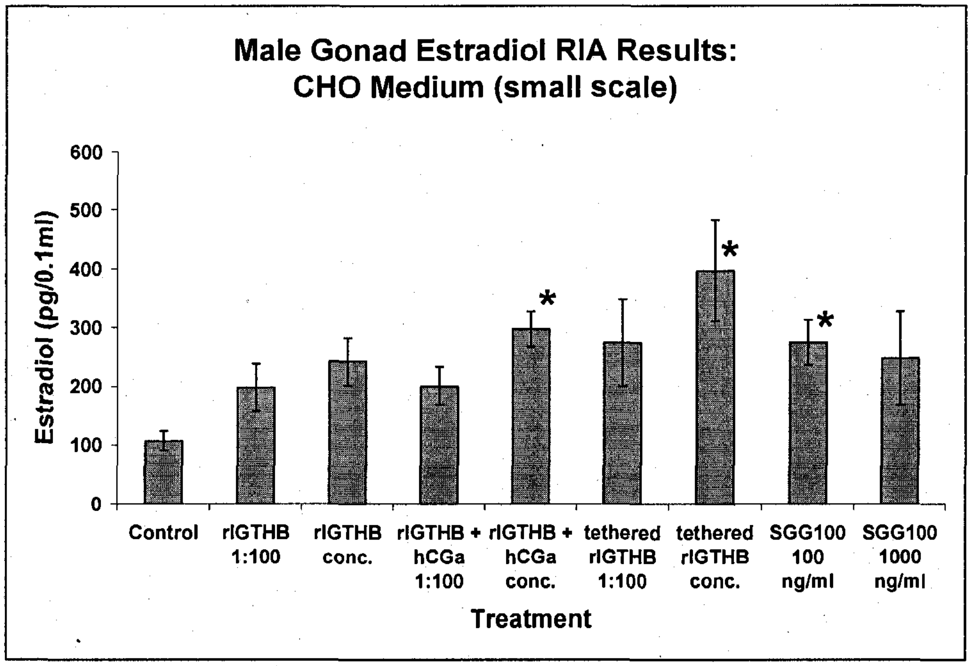
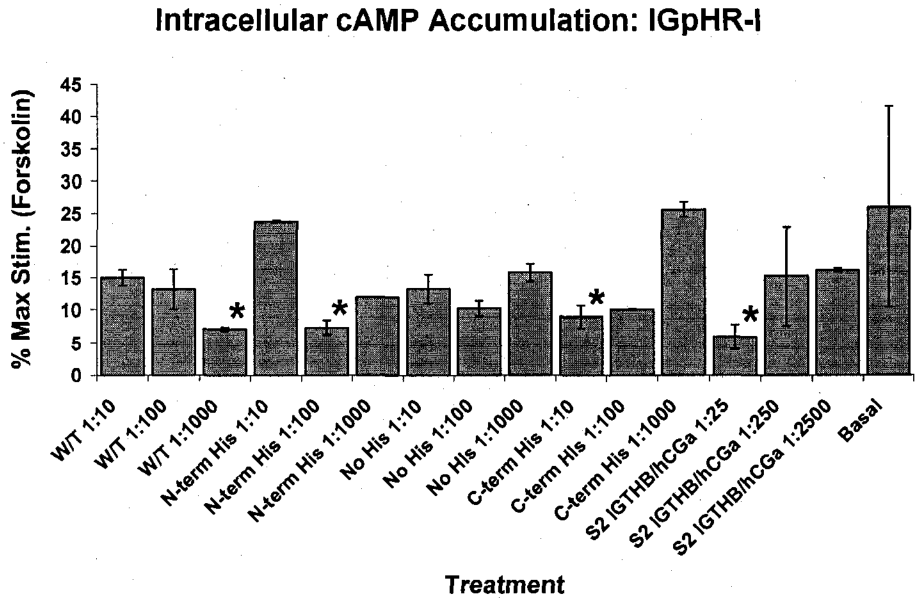


Figure 15: The graph above illustrates the response of male lamprey gonadal tissue to various treatments of concentrated stable transfected CHO cell growth medium. There was significant response to the concentrated medium from the tethered lGTH-β/hCGα treatment, which was higher than that of the SG-G100 positive control ($P>0.05$), as well as a 3-fold difference compared to the HBSS control ($P<0.05$). The response from recombinant lGTH-β cotransfected CHO medium was also significantly higher than the control ($P<0.05$).

In-Vitro Culture- Cos-7 cAMP Receptor Assay

A total of 3 concentrated medium samples from purified stable CHO cell lines were tested for activity. CHO purified medium samples were tested for activity with previously purified tethered lGTHβ/hCGα from S2 cells using cAMP accumulation in Cos-7 cells (Figure 16). Hormone activity was measured against the maximum cAMP stimulation achieved through Forskolin treatment, and compared to transfection of Cos-7 cells with lGpHR-I vector (Graph 1, Figure 16) to the blank vector construct (Graph 2, Figure 16). None of the purified medium samples appeared to induce any response using the lGpHR-I receptor in Cos-7 cells. All measured cAMP levels for treatments were lower or equal to the basal level of cAMP in the cell. Several of the recombinant treatments for the transfected receptor were significantly lower than the basal levels of cAMP ($P<0.05$).

In order to verify the receptor response to the ligand, a second cAMP EIA was completed using rat LH-R transfected into Cos-7 cells, and treated with oLH as a positive control for activity. There was a significant measurable response to oLH in the cells transfected with Rat LH-R ($P<0.05$) (Graph 1, Figure 17). The response was 3-fold higher than basal, and was solidified by the lack of activity of the same treatment with cells transfected with the blank construct (Graph 2, Figure 17)



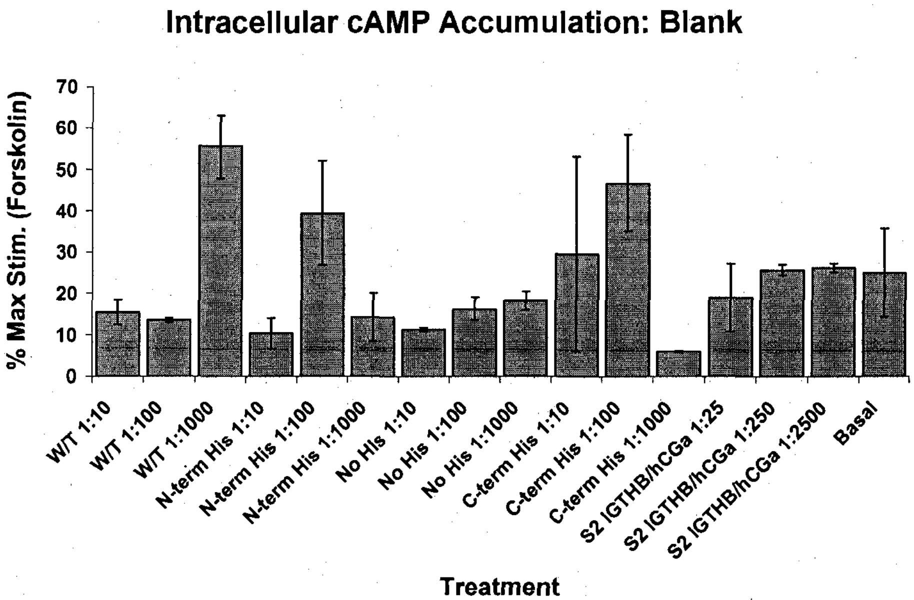


Figure 16: The response of lGpHR-I receptor in Cos-7 cells was measured in-vitro using the cAMP accumulation EIA kit (GE Lifesciences, 2008). Levels of cAMP accumulation were compared to the maximal stimulation from Forskolin treatment, as well as basal levels of cAMP in the cell. There was no significant response to any of the constructs against the lGpHR-I transfection in Cos-7 cells ($P>0.05$). In many cases, blank construct cAMP levels were higher than lGpHR-I transfected cells.

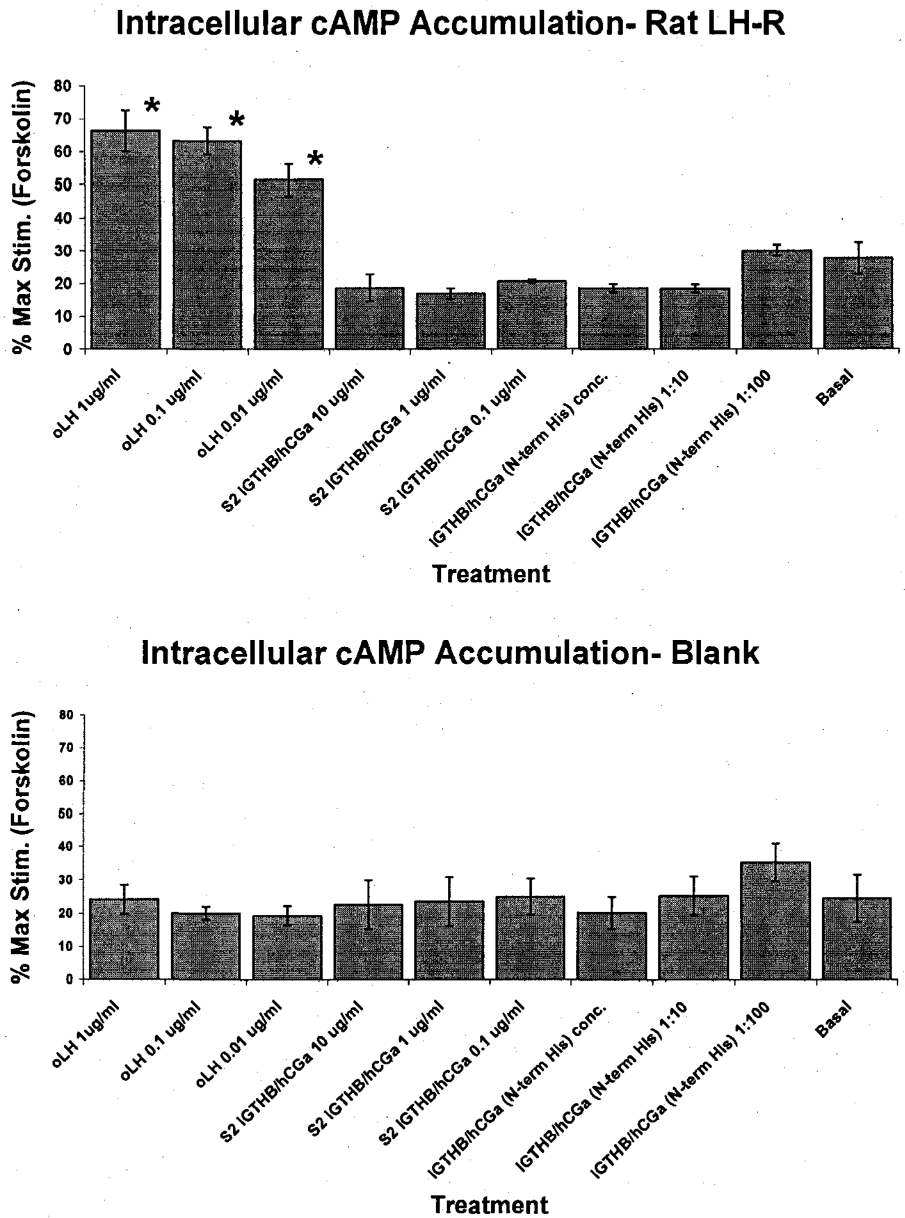


Figure 17: The receptor stimulation in the cAMP accumulation EIA (GE Lifesciences, 2008) was verified using Rat LH-R transfected into Cos-7 cells and oLH as a positive control. Response of LH-R to oLH was 3-fold higher than basal ($P<0.05$). None of the other concentrated CHO medium treatments showed any significant cAMP response to Rat LH-R that was higher than basal.

DISCUSSION

This study has identified that the lamprey gonadotropin beta can be produced using the DES expression system, although with lower quantities than expected. Channel catfish LH and FSH were isolated in much higher quantities of 6-8 mg/L using the DES system, but as cited by the authors, the higher yield was due to the presence of combined alpha and beta subunits (Xing et al., 2004). These authors proposed that the quality of this high yield of recombinant channel catfish GTH was due to the successful expression of the ccGTH-α subunit, allowing the ccGTH-β subunit to be excreted readily into the medium. Since the lamprey GTH-α subunit has not yet been identified, the only option at this time was to try and develop an active lGTH-β, incorporating a different alpha subunit from another vertebrate species. Attempts at combining the lamprey GTH-β with other subunits using co-expression in S2 cells did not appear to help with protein yield. In several stable transfections with ccGTH-α and hGpH-α, the GTH-β was expressed and detected through RT-PCR but was not excreted or produced as a functional protein from the cell.

Using known techniques to force confirmation of the lGTH-β with other alpha subunits, prepared purified samples of tethered lGTH-β/hCG-α were produced from S2 cells and CHO suspension cultures, with similar problems of low protein yield. Enough protein was collected and purified using Nickel Sepharose in order to test the recombinant protein activity against the lamprey GpHR-I. Results from cAMP accumulations did not correlate well with previous lamprey receptor activity studies (Freamat et al., 2008), and showed little or no response to receptor transfected Cos-7 cells.

The current study showed that the recombinant lamprey GTH beta was produced at lower quantities than expected. Even with the various combinations of other alpha subunits, tethered or cotransfected, there was no significant activity from any of the purified samples. The original hypothesis was that the individual lGTH-β subunit was playing a role in lowering the amount of total protein that could be produced from the cell. Since then, the use of the individual subunit was eliminated by tethering two subunits together, and yet there was still a lack of high production of protein from the cells. Differences in the loop region required for subunit overlap between the lGTH-β and hCG-α or ccGTH-α may cause protein conjugation to be more difficult or impossible (Sower et al., 2006).

The data to date suggest that lampreys may only have one glycoprotein hormone (Sower et al., 2006), although the possibility of not having an alpha subunit is low, since both alpha and beta subunit for a hagfish (a sister group to lamprey) gonadotropin have been identified (Nozaki et al., 2005) and a partial alpha-like subunit has been identified from the lamprey genome (Wilmot et al., unpublished).

Whether or not a recombinant lamprey GTH-β can be functional will require further studies. The production methods used to try to produce the hormone in various ways were unsuccessful in obtaining a high yield product. Based on recombinant GTH studies in other vertebrates, it is likely that the lamprey GTH alpha will be required in order to conform to the beta subunit for full functional activity. In all other vertebrate species, the alpha subunit of a glycoprotein hormone as previously discussed is essential in producing a functional hormone. The lGTH-β most likely requires the addition of the proper alpha subunit to fold properly and to be released from the cell, allowing binding to the lamprey glycoprotein hormone receptor (Salesse et al., 1975). Another possibility is that the Nickel Sepharose purification may alter the structure of the beta subunit, leading to a modified glycosidic residue or a protein incapable of proper folding. The activity of the glycoprotein hormone is directly linked to glycosylation (Trout et al., 1999); therefore until the lamprey GTH-α is identified, the reason for the lack of function of a tethered heterologous hormone remains unknown.

In this study, several molecular methods were used to produce stable lines from two cell types (S2 and CHO) that produced recombinant lamprey gonadotropin beta subunit. The methods for histidine tagged protein purification and medium concentration were optimized, and the procedure was easily reproducible. A consistent amount of protein was purified from the DES induced expression, although the yield was much lower than originally expected.

Production of protein from CHO cells was not as easily obtained. Mammalian and non-mammalian cell lines were capable of becoming stable through selection with Geneticin G418 antibiotic and appeared to express the hormone as indicated by RT-PCR, but did not yield an active purified protein with large scale growth of CHO cell suspension cultures. The loss of protein production could be due to the manipulation of a once adherent cell line which was forced to grow under suspension growth conditions at high density. This alone can alter the cellular activity of the cell, and change the composition of proteins that were once expressed to allow for the cell survival in suspension (Galbraith et al., 2006). Future work with CHO cells should be optimized for suspension culture conditions before transfection of recombinant protein constructs.

The results from this study can be used to develop procedures for the production of recombinant lamprey gonadotropins, especially once the alpha subunit has been identified. There are also other cell system methods that should be tested, such as the use of Baculovirus expression or the utilization of highly modified yeast cultures that can yield the specific types of glycosylation necessary for protein function. The protocols and methods from this study can now be used as a basis for future studies in the production of recombinant lamprey gonadotropin.

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APPENDIX

IACUC Approval

The research described herein falls under the approval of Neuroendocrine Control of Reproduction to Dr. Stacia Sower, shown below.

University *of* New Hampshire

Research Conduct and Compliance Services, Office of Sponsored Research
Service Building, 51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

23-May-2007

Sower, Stacia
Biochemistry Molecular Biology, Rudman Hall
Durham, NH 03824

IACUC #: 070401
Project: Neuroendocrine Control of Reproduction/Training of Undergraduate & Graduate Students
Category: C
Approval Date: 23-May-2007

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.* The IACUC made the following comment(s) on this protocol:

1. Eileen Balz needs to complete the Medical Questionnaire prior to handling any vertebrate animals.
2. The investigator needs to correct the larval sea lamprey numbers in Section V, Table 1 and update the total animal number (and in other places in the form, if necessary).

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,
Jessica Bolker
Jessica A. Bolker, Ph.D.
Chair

cc: File